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Phenotypic Differences in Angelman Syndrome Patients: Imprinting Mutations Show Less Frequently Microcephaly and Hypopigmentation Than Deletions

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Angelman syndrome (AS) is a relatively frequent disorder of psychomotor development caused by loss of function of a gene from chromosome 15q11–q13, a region subject to genomic imprinting. The AS gene(s) is exclusively expressed from the maternal chromosome. Several kinds of mutations have been found to cause AS. More than half of the cases exhibit a deletion of the maternal 15q11–q13 region. Recently, we and others described a new mutation type, the imprinting mutation, characterised by normal, biparental inheritance but aberrant methylation patterns of the entire chromosomal region. In AS, a paternal imprint is found on the maternal chromosome probably leading to functional inactivation of the AS gene(s). We have now compared the phenotype of 9 AS patients with imprinting mutation to that of nine age-matched ones with a maternally derived deletion. Both groups were evaluated for 19 common AS symptoms. All patients, independently of their molecular findings, showed classical AS symptoms such as mental retardation, delayed motor development, and absent speech. In contrast, for two signs, hypopigmentation and microcephaly, a different distribution among both groups was observed. Only one of nine AS patients with an imprinting mutation, but seven of nine in the deletion control group showed either symptom. Our results suggest that imprinting mutations, in contrast to deletions, cause only incomplete loss of gene function or that maternally derived deletions affect also genes not subject to genomic imprinting. We conclude that AS is caused by loss of function of a major gene that is imprinted but that there are also other genes that contribute to the phenotype when in hemizygous condition.

KEY WORDS: Angelman syndrome, Prader-Willi syndrome, imprinting mutation, genomic imprinting, microcephaly, oculocutaneous albinism (OCA2), chromosome 15

INTRODUCTION

Angelman syndrome (AS) is a relatively frequent disorder of mental and motor development. Affected individuals invariably show severe mental retardation, delayed motor development, almost complete absence of speech, and movement or balance disorder, usually ataxic gait. Most patients also present a typical face with wide and open mouth, protruding tongue, and prominent chin, and their behaviour is often characterised by frequent laughter and inappropriate happiness. Furthermore, seizures and characteristic EEG findings are found in most children [Williams et al., 1995]. Its frequency has been estimated to be 1 in 16,000 newborns in the urban area of West Berlin [Reis et al., 1994a].

The genetic basis of Angelman syndrome is complex. Findings of cytogenetically visible deletions have localised the AS gene to chromosome 15q11–q13 [Kaplan et al., 1987; Magenis et al., 1987], a region also containing the locus of the clinically distinct Prader-Willi syndrome (PWS). Knoll et al. [1989] have shown these two syndromes to result from similar deletions but to differ in parental origin of the deletion. AS results from the deletion of the maternal chromosome, whereas PWS correlates with loss of the paternal allele. This and analogous observations in cases with uniparental disomies have led to the conclusion that the region 15q11–q13 is genomically imprinted and that the AS gene(s) is expressed exclusively from the maternal chromosome, whereas the paternal AS gene(s) is inactive [for review see Nicholls, 1993].

The cause of AS is the loss of function of the AS gene(s), which is expressed only from the maternal

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chromosome. Several paternally expressed genes from 15q11–q13 have been identified [Reed and Leff, 1994; Nakao et al., 1994; Sutcliffe et al., 1994; Wevrick et al., 1994], but cloning of a maternally expressed one has remained elusive so far. At present it is not known whether all symptoms in AS are caused by the loss of function of a single gene or whether several genes are involved. Different genetic mechanisms can result in the loss of function of the AS gene(s). Approximately two-thirds of the patients show a deletion of roughly 4 million basepairs of the maternal chromosome 15q11–q13, which sometimes can be seen microscopically [Saitoh et al., 1994]. Whereas uniparental disomies (UPD), i.e., both chromosomes of paternal origin, are relatively rare, a large group of patients with typical AS have no detectable alteration at the molecular level. They might have very small deletions or point mutations affecting the AS gene(s). Finally, a new mutation type, the imprinting mutation, has recently been described, which is characterised by an aberrant methylation pattern of the maternal chromosome in Angelman syndrome. The AS/PWS region bears several loci of parent-of-origin-specific methylation. These patients have biparental inheritance but on both chromosomes a paternal methylation pattern. Likewise, patients with PWS due to imprinting mutations exhibit a maternal imprint on the paternal chromosome [Glenn et al., 1993; Reis et al., 1994b; Buiting et al., 1995].

Methylation is well known to be a regulator of the transcriptional activity of genes [Busslinger et al., 1983; Bird, 1986; Yeivin and Razin, 1993]. The aberrant methylation pattern seen in AS individuals with imprinting mutations is probably the correlate of erroneous transcriptional inactivation of their maternal AS gene(s). In some of these imprinting mutation patients, deletions, 1 MB proximal to the AS gene(s) critical region, have been described in *cis* [Sutcliffe et al., 1994; Buiting et al., 1995]. These authors proposed a mechanism where methylation would be regulated by a structure from those deleted regions, the "imprinting centre." It must be noted that mechanisms in *trans* [Reis et al., 1994b] are not yet excluded from being responsible, at least partially, for imprinting mutations.

In our laboratory we have been able to identify nine cases of AS patients with imprinting mutations including one sibship among 450 investigations for AS. The novelty of the imprinting mutation led us to the question of whether it results in the same phenotype as other mutations. The purpose of this study was to investigate whether AS patients with imprinting mutations have the same clinical signs and symptoms as an age-matched control group with deletions.

MATERIALS AND METHODS

Molecular Investigations

Methylation of patient and parent DNA at the locus D15S63 was investigated by Southern blot analysis with probe PW71B using the methylation sensitive enzymes HpaII or CfoI as described [Dittrich et al., 1992]. Biparental inheritance of the AS/PWS region was demonstrated by microsatellite analysis. Genotypes of patients and their parents were assessed at GABRB3 [Mutirangura et al., 1992], D15S10 [Lindeman et al., 1991]/D15S210 and D15S128 [Gyapay et al., 1994] as described elsewhere [Reis et al., 1994; Bürger et al., in prep.]. These loci are located within the AS/PWS deletion region and flank the AS gene(s) critical region proximally (D15S10 and D15S210) and distally (GABRB3) [Reis et al., 1993; Greger et al., 1994; Malcolm and Donlon, 1994]. D15S128 is very close to the putative imprinting centre.

Clinical Investigation

The patients described in this study have been referred to us from all over Germany and live in Germany. Of those with imprinting mutations, six are of German and three of Yugoslav origin. Seven of the deletion patients are German, one is Italian, and one is Thai. Prior to molecular investigations, a questionnaire was completed by the paediatricians who are managing the patients. This questionnaire asks for the presence of 19 common symptoms of the Angelman syndrome: mental retardation, delayed motor development, inappropriate happiness, feeding problems during infancy, absent speech, normal pregnancy, brachycephaly, microcephaly, prominent chin, macrostomy, protrusion of the tongue, hypopigmentation, ataxia, seizures, restlessness, sleeping problems, hypotonia of the trunk, hypertonia of the extremities and typical EEG-findings. Subsequently, all of the nine patients with an imprinting mutation were seen and investigated together with their mothers by at least one of the authors. Symptoms were evaluated by clinical examination. Hypopigmentation was evaluated by comparison of patients skin, hair, and iris pigmentation with that of their parents. Microcephaly was defined as a head circumference lower than the mean value minus 2 standard deviations [Gorlin et al., 1990].

Statistical Analysis

Since the phenotypic expression of AS is age-dependent, for each patient with an imprinting mutation an age-matched patient with a maternally derived deletion was sought. For both groups of patients, the frequencies of the symptoms were counted. To assess significance for a different distribution of the frequencies of a particular symptom Fisher's exact test was calculated. If data about the presence of a symptom in a deletion patient were not available, the age-matched imprinting mutation patient was also excluded from the calculation (values given in brackets in Table I). The P values have been calculated for the given and a more extreme distribution of the AS signs. The null hypothesis—frequencies of respective symptoms in imprinting mutation and deletion patients are different—is rejected at the 5% level ($P \geq 0.05$).

RESULTS

Molecular Investigations

Results of molecular investigations are given in Table I. Our microsatellite data show biparental inheritance of the AS/PWS region in all patients. The methylation sensitive Southern blot with probe PW71B (D15S63) demonstrated absence of the fragment with

TABLE I. Methylation Status at D15S63 (PW71B) and Genotypes at D15S128, D15S10/D15S210, and GABRB3*

Family	Locus	Probe	Father	Mother	Patient 1	Patient 2
W	D15S128	MS	3–5	3–4	3–4	
	D15S10	3–21	1–3	2–2	2–3	
	GABRB3	MS	5–11	2–10	2–11	
	D15S63	PW71B	1–2	1–2	2–2	
D	D15S128	MS	1–2	3–3	1–0	2–0
	D15S10	MS	1–2	1–2	1–2	1–1
	GABRB3	MS	4–11	4–11	11–11	4–11
	D15S63	PW71B	1–2	1–2	2–2	2–2
Kl	D15S128	MS	7–8	7–7	7–7	
	D15S210	MS	8–8	5–8	8–8	
	GABRB3	MS	2–10	3–9	3–10	
	D15S63	PW71B	1–2	1–2	2–2	
Ge	D15S128	MS	1–5	3–3	1–3	
	D15S10	MS	1–1	2–2	1–2	
	GABRB3	MS	5–6	8–10	5–10	
	D15S63	PW71B	1–2	1–2	2–2	
Ki	D15S128	MS	6–11	5–8	5–6	
	D15S210	MS	6–9	6–8	6–8	
	GABRB3	MS	5–7	3–12	5–12	
	D15S63	PW71B	1–2	1–2	2–2	
La	D15S128	MS	1–6	6–9	6–6	
	D15S210	MS	2–4	6–6	4–6	
	GABRB3	MS	2–10	4–11	2–4	
	D15S63	PW71B	1–2	1–2	2–2	
Gr	D15S128	MS	7–7	7–8	7–7	
	D15S210	MS	1–7	5–7	7–7	
	GABRB3	MS	10–11	5–11	5–10	
	D15S63	PW71B	n.d.	n.d.	2–2	
Le	D15S128	MS	3–5	4–4	4–5	
	D15S210	MS	4–8	1–7	1–8	
	GABRB3	MS	11–11	5–8	8–11	
	D15S63	PW71B	1–2	1–2	2–2	

*D15S63, PW71B: 1 represents the maternal allele; 2 the paternal one; n.d.: not determined; MS: microsatellite.

maternal methylation imprint in all patients. All parents investigated showed normal biparental methylation patterns. Two siblings (Family D) have a small deletion of their maternal chromosome including microsatellite locus D15S128 (Table I). The exact extent of the deletion is described in Buiting et al. [1995] ("Family D") and is supposed to include the putative imprinting centre. No other alterations have been found in any of the other patients so far.

Clinical Investigations

The data from our clinical investigations are presented in Table II. All patients show typical AS symptoms, such as severe mental retardation, absent speech, and delayed motor development, independently on the underlying molecular defect. All meet the diagnostic criteria for AS, as recently proposed [Williams et al., 1995]. Nevertheless, for two symptoms, we observed a different distribution among both groups. Only one of nine imprinting mutation patients, but seven of nine deletion patients showed microcephaly and only one of nine patients with imprinting mutation was hypopigmented as compared to seven of nine with maternally derived deletion ($P = 0.008$).

Head circumferences of all patients are plotted in Figure 1. Whereas values for imprinting mutation patients are mostly around the mean, those from patients with deletions are typically slightly below the −2 standard deviations line. Interestingly, brachycephaly, which, like microcephaly, is a correlate of skull bone formation, was found to be equally frequent in both groups. Two of the imprinting mutation patients are siblings, a boy and a girl of Yugoslav origin, aged 15 and 13 years, respectively. Although both have the same mutation, the boy is microcephalic and the girl is not.

The other 17 AS symptoms investigated in this study are not differently distributed among both groups (Table II).

DISCUSSION

To study the phenotypic consequences of imprinting mutations, we have compared a group of AS patients with imprinting mutations to an age-matched control group of AS patients with large, maternally derived deletions. Both types of mutation give rise to typical AS. For 17 AS symptoms, our study showed no significant differences between the two groups. In contrast, we found two symptoms, microcephaly and hypopigmentation, to be significantly less frequent in the group of imprinting mutation patients.

TABLE II. Frequencies of the Respective Symptom in Group of Patients With Either Imprinting Mutation or Deletion

Symptom	Imprinting mutation	Deletion	P^*
Severe mental retardation	9/9	9/9	
Delayed motor development	9/9	8/8	
Inappropriate happiness	9/9	7/9	0.24
Feeding problems in infancy	4/9 (4/8)	6/8 (6/8)	0.30
Absent speech	9/9	9/9	
Complicated pregnancy or birth[a]	3/9	3/9	
Brachycephaly	4/9 (3/8)	5/8 (5/8)	0.31
Microcephaly	1/9	7/9	*0.008*
Prominent chin	1/9	5/9	0.07
Wide and open mouth	8/9	8/9	
Protruding tongue	6/9	6/9	
Hypopigmentation	1/9	7/9	*0.008*
Ataxic gait	8/9	8/9	
Seizures	6/9	7/9	
Restlessness	7/9 (5/7)	7/7 (7/7)	0.23
Sleeping problems	8/9 (7/7)	4/7 (4/7)	0.10
Hypotonia of the trunk	4/7 (3/5)	7/7 (5/5)	0.22
Hypertonia of the extremities	6/9 (5/7)	6/7 (6/7)	0.50
Typical EEG findings	6/8 (6/8)	8/9 (7/8)	0.50

[a] These are unspecific and relatively frequent complications (reduced movements of the child, preterminal labour, birth by sectio) that did not differ between the two groups.
* Calculated as described in Materials and Methods. If data about the presence of a symptom in a deletion patient were not available, the age-matched imprinting mutation patient was also excluded from the calculation (values given in brackets).

Phenotypes of Imprinting Mutations Equal Those of Deletions

Since both groups present similar phenotypes, we conclude that the amount of functional AS gene(s) product should be similar, too. In case of deletion the loss of gene function is complete, and therefore the suppression of the AS gene(s), caused by imprinting mutations, should be almost complete as well. This is not unexpected since the AS gene(s) of the paternal chromosome also is totally suppressed by the same mechanism, i.e., probably methylation. Therefore, our clinical data indicate that imprinting mutations result in an epigenetic state that completely resembles that of the oppositely imprinted chromosome. These conclusions are supported by recent findings that erroneous biallelic methylation of the p16 tumour-suppressor gene also results in tumours, the same as deletions of this gene [Merlo et al., 1995]. If an imprinting mutation leads to a paternal methylation pattern of the maternal chromosome, one would expect the same consequences that result from uniparental disomy. This conclusion could be investigated by comparing the phenotypes of imprinting mutations to those of uniparental disomies. No AS patients with UPD were available to us for investigation. Whether milder types of imprinting mutations might lead to an intermediate phenotype of methylation and gene expression has to be investigated in the future as well. Familial nondeletion AS patients without aberrant methylation pattern are assumed to bear small mutations in the AS gene(s). Saitoh et al. [1994] showed that all such AS patients present the major AS symptoms as mental and motor retardation, absence of speech, and ataxia. This does not exclude more than one gene being responsible for the AS phenotype, but it indicates that these genes have to be close together.

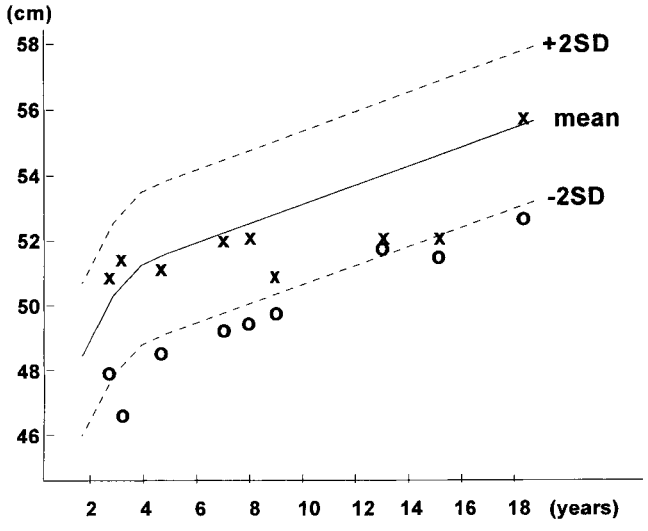

Fig. 1. Head circumferences of AS patients investigated: patients with imprinting mutations are indicated by a cross (x), age-matched deletion patients by circles (o). Mean, +2, and −2 standard deviation lines (+2SD and −2SD) are given [Gorlin et al., 1990]. Only one imprinting mutation patient presents a head circumference below the −2SD value, vs. seven of nine deletion patients.

Microcephaly Is Rare in Imprinting Mutation Patients

Microcephaly might result from another, not imprinted gene, which is in proximity to the imprinted

major AS gene and affected by deletions but not imprinting mutations. Evidence for this hypothesis comes from two other investigations. Saitoh et al. [1994] have measured the head circumferences in AS individuals with and without deletion and found those with deletion to be 0.6 SD below nondeletion patients. However, they observed the lowest head circumferences in familial nondeletion (−2.6 SD) and deletion cases (−2.4 SD), whereas sporadic cases were much less microcephalic (−1.6 SD with and −1.0 SD without deletion). These findings could be explained by an additional effect of mutated but present AS protein(s) compared to absent one(s). Bottani et al. [1994] suggested a milder phenotype in AS with uniparental disomy. Both of their patients did not present either microcephaly or hypopigmentation. These data support a model where more than one gene would be responsible for the phenotype of deletion patients, whereas only imprinted gene(s) would be suppressed by an imprinting mutation. Because of its high recombination frequency, which is more than twice of the average recombination rate, the 15q11–q13 region is assumed to be very generich [Robinson and Lalande, 1995]. Hence, the deletion may affect >100 genes. At least some of them should result in some phenotypic alterations, even in the heterozygote state.

Another possible mechanism to explain the different distribution of microcephaly could be gene dosage. If growth of the head were very sensitive to AS gene(s) dosage and the imprinting mutation did not completely suppress its expression, then imprinting mutation patients would have bigger brains and thus larger skull volumes. The same phenotypical consequences would result if the AS gene(s) is not regularly imprinted in all tissues or at all developmental stages.

Finally, we have not observed a high degree of correlation of phenotype with genotype. Among the imprinting mutation patients described here is a pair of siblings with a deletion at the putative imprinting centre near locus D15S128 which is several hundred kb proximal to the AS gene(s) critical region [Buiting et al., 1995]. Both siblings have the same maternal haplotype with the deletion but different paternal ones [haplotypes published as "Family D" in Reis et al. 1994b]. Although both have the same mutation, one is microcephalic and the other is not. This also indicates that head circumference is not determined by suppression of an imprinted AS gene(s), but rather by another gene(s).

HYPOPIGMENTATION

Our finding that hypopigmentation is distributed differently is not surprising because in AS and PWS it was almost exclusively found in cases with deletions. Only approximately half of nondeletion patients show hypopigmentation [Saitoh et al., 1994]. The P gene, which is located within the AS/PWS deletion and is not subject to genomic imprinting, is the cause of autosomal recessive oculocutaneous albinism (OCA2) [Lee et al., 1994]. In AS and PWS, hypopigmentation is correlated with deletions of one copy of this gene [Brilliant et al., 1994]. A small subset of AS and PWS patients who also have OCA2 exhibit a deleterious mutation on their second P gene allele. Our findings of normal pigmentation in AS patients with imprinting mutations confirms that the P gene is not subject to genomic imprinting.

In summary, the results of our investigation show that imprinting mutations give rise to typical AS. This indicates that the imprinting mutation suppresses the AS gene(s) completely. However, hypopigmentation and microcephaly occur significantly more frequently in patients with deletions. Whereas hypopigmentation seems to be caused by the loss of one copy of the P gene, the different distribution of microcephaly remains unexplained. We conclude that AS is caused by loss of function of a major gene that is imprinted but that there are also other genes that contribute to the phenotype when in hemizygous condition.

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